For Reference

NOT TO BE TAKEN FROM THIS ROOM

Ex libris
universitatis
albertaensis

Digitized by the Internet Archive
in 2019 with funding from
University of Alberta Libraries

https://archive.org/details/Johansen1977

THE UNIVERSITY OF ALBERTA

RELEASE FORM

NAME OF AUTHOR . . . Eric Johansen . . . .

TITLE OF THESIS . . . Study of an unstable reversion of the *gal3* insertion of *E. coli* . . . .

DEGREE FOR WHICH THESIS WAS PRESENTED . . . Master of Science . . . .

YEAR THIS DEGREE GRANTED . . . 1977 . . . .

Permission is hereby granted to THE UNIVERSITY OF ALBERTA LIBRARY to reproduce single copies of this thesis and to lend or sell such copies for private, scholarly or scientific research purposes only.

The author reserves other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without the author's written permission.

THE UNIVERSITY OF ALBERTA

STUDY OF AN UNSTABLE REVERSION OF THE *gal3* INSERTION OF *E. coli*

by

© ERIC JOHANSEN

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF GENETICS

EDMONTON, ALBERTA

FALL, 1977

MF-81

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled Study of an unstable reversion of the *gal3* insertion of *E. coli* submitted by Eric Johansen in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

Spontaneous mutations normally occur at low (10^{-5}-10^{-6}) frequencies. A system is described where mutations seem to occur at an exceptionally high rate (10^{-2}-10^{-3}) at a localized region, and a mechanism for this kind of genetic instability is proposed.

The *gal3* mutation of *E. coli* was caused by the insertion of an IS*2* element in the operator-promoter region of the *gal* operon. This mutation reverts spontaneously by excision or partial deletion of IS*2* to produce inducible or constitutive revertants, which are stable. Unstable revertants, which constantly segregate gal^- colonies and show low levels of constitutive enzyme synthesis, arise at a frequency of 10^{-10}. These reversions have been reported to arise by inversion of IS*2*, or by duplication of the *gal* operon.

An unstable reversion ($gal^c 331$), isolated from a *gal3* $recA^-$ strain, has been studied. This reversion produces gal^- segregants at a high rate (9×10^{-3} segregants/cell/division). Electron microscopy of DNA heteroduplexes of λgal phages bearing $gal^c 331$ revealed that the unstable reversion does not cause any visible change in the orientation or length of the IS*2*. Similarly, no change was observed in the *gal* operon. The results eliminate the traditional explanations of genetic instability, viz., plasmid formation, tandem duplication, or inversion of IS*2*. Instead, these reversions seem to arise by double mutations which inactivate the transcription termination site on IS*2*. Instability is apparently

dependent upon (i) the presence of a unique sequence provided by IS*2*, and (ii) the occurrence of transcription along it.

The segregants produced by *gal*^{c}*331* are heterogeneous, and include not only *gal*$^{-}$ but also *gal*^{wc} (weak-constitutive). Neither kind shows any visible change in IS*2* or the *gal* operon by electron microscopy. Our interpretation is that these segregants arise by mutations occurring at an unusually high (10^{-2}-10^{-3}) rate. The high mutability is attributed to errors introduced during the repair of specific lesions formed along IS*2* during transcription. Experiments involving various treatments which enhance DNA repair, and repair mutations, support this interpretation. Evidence that these lesions might be initiated from single strand nicks is provided by experiments with a temperature sensitive ligase mutant. It is suggested that IS*2* can act as a potential hot-spot for repair.

The site for the initiation of transcription in constitutive revertants of *gal3* has been determined. Experiments with adenyl cyclase and cyclic AMP receptor protein mutants suggest that, in these revertants, *gal* transcription is initiated at a promoter located on the IS*2* element rather than at the normal promoter for the *gal* operon.

ACKNOWLEDGEMENTS

I wish to thank my supervisor, Dr. A. Ahmed, for his support, encouragement and assistance throughout the course of this study.

I also wish to thank Chris Somerville for many interesting discussions and helpful suggestions. The efforts of Roger Bradley and Dr. D. Scraba (Department of Biochemistry) during electron microscopy are greatly appreciated. I am also grateful to Dr. C. Strobeck for deriving the formula for calculating high mutation rates.

My parents, and my wife, Karen, are thanked for their moral support and encouragement.

This work was supported by a National Research Council of Canada Student Fellowship.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

A new class of genetic elements, called IS-elements (IS = Insertion sequence), has recently been the subject of extensive study (reviewed by Starlinger & Saedler, 1976; Kleckner, 1977). IS-elements are discrete DNA sequences of defined lengths that are present in multiple copies in the chromosomes and plasmids of several gram negative bacteria. These elements are usually detected when they are translocated to new positions on the chromosome. When integrated into bacterial operons, they abolish the function of the gene into which they are integrated and severely depress transcription of genes located downstream in the direction of transcription. Insertion of IS-elements causes extreme polarity with a 100- to 1000-fold reduction in expression of distal genes. In contrast, classical polar mutations (such as nonsense mutations) cause only a 3- to 10-fold reduction in gene expression. Insertion mutations map as point mutants and cannot be induced to revert by known mutagens although most revert spontaneously.

The F-factor contains a number of IS-elements located in defined regions of its chromosome. It was found that integration of the F-factor into the bacterial chromosome, during the production of Hfr strains, is facilitated by recombination between the IS-elements on the two genomes involved (Starlinger & Saedler, 1976).

Mutations in *E. coli* K12 caused by IS-elements have been

identified in the *gal*, *lac* and other operons. Bacteriophage λ mutants and variants have been shown to contain IS-elements, although wild type λ does not normally harbor these elements. Several plasmids known to confer antibiotic resistance have been shown to carry different insertion sequences. IS-elements have also been identified in *Salmonella typhimurium* and *Citrobacter freundii*.

The IS-elements are divided into classes based on their sizes and sequences. IS*1* is approximately 800 base pairs in length and is polar when integrated in either orientation. IS*2* is 1370 base pairs in length. All polar IS*2* mutations isolated to date have the insertion sequence integrated in the orientation designated I. Recent evidence suggests that IS*2* in orientation II is non-polar (Mosharrafa *et al.*, 1976). IS*3* and IS*4* are each 1400 base pairs in length but have no sequence homology. Several insertion mutations are caused by IS-elements not yet identified as belonging to one of these four classes. It is likely that new classes of IS-elements will be found among these mutations.

The unusual properties of the *gal3* mutation (Lederberg, 1960) of the *gal* operon of *Escherichia coli* have been the subject of several studies (reviewed by Ahmed, 1977). *gal3* is a mutation of spontaneous origin which maps as a point mutant in the operator-promoter (*OP*) region of the *gal* operon (Shapiro & Adhya, 1969). It reverts spontaneously but fails to respond to various chemical mutagens (Adhya & Shapiro, 1969). It exhibits extreme polar

effects on the synthesis of *gal* mRNA and the three enzymes (viz., kinase, transferase, and epimerase) coded by the *gal* operon (Hill & Echols, 1966). This polarity, however, is not relieved by the action of nonsense translational suppressors (Adhya & Shapiro, 1969). The only external mutations known to suppress *gal3* cause alterations of the rho factor (Das *et al.*, 1976) which is required for the termination of transcription. It has been shown that the *gal3* mutation arose by the linear insertion of an approximately 1.1 - 1.2 kb† DNA sequence into the *galOP* region (Ahmed & Scraba, 1975). This insertion has recently been identified as IS*2* in the polar orientation (I) (Fiandt *et al.*, 1977). The extreme polarity of *gal3* seems to be due to the presence of a sequence recognized by the rho factor on IS*2* (de Crombrugghe *et al.*, 1973). The ends of the insertion act as preferred sites for the formation of extended deletions (Ahmed & Johansen, 1975; Ahmed & Scraba, 1977).

The most intriguing feature of the *gal3* mutation is the production of three different kinds of gal^+ revertants (Hill & Echols, 1966; Morse, 1967; Morse & Pollock, 1969). The most common class consists of genetically stable revertants, which are inducible for the *gal* operon and resemble the gal^+ wild type in all respects. Another class includes revertants which are stable but exhibit a high level of constitutive enzyme synthesis comparable to a fully-induced wild type. The least frequent class consists of revertants which are unstable and show constitutive enzyme synthesis at a relatively low level. These revertants

typically segregate *gal*$^-$ colonies at a high rate (10^{-2}-10^{-3} segregants/cell/division). Both kinds of constitutive revertants are transduced with very poor efficiency by bacteriophage λ. Similar reversion behavior has been reported for another IS*2* mutation (*308*) located in the *OP* region of the *gal* operon (Saedler *et al.*, 1974). Employing various genetic and physical techniques, it has been shown that the stable inducible revertants arise by precise excision of IS*2* leading to the restoration of the original *gal*$^+$ base sequence (Ahmed, 1975). A stable constitutive reversion was found to have arisen by deletion of a portion of the IS*2*, probably by the removal of the rho-sensitive site (Ahmed & Johansen, 1975).

The nature of the unstable constitutive revertants, however, has remained obscure and controversial. According to one proposal, these reversions are caused by inversion of the IS*2* (Saedler *et al.*, 1974). The insertion is believed to harbor a promoter which, in one orientation (designated I), interferes with *gal* transcription to produce the *gal*$^-$ phenotype. In the opposite orientation (II), the same promoter causes constitutive *gal* transcription. This hypothesis does not adequately account for the fact that a *gal3* strain, AD1600, defective in transcription termination factor rho, is constitutive for galactokinase (Das *et al.*, 1976). An alternative proposal is that these reversions arise by tandem duplications of the *gal* operon (Ahmed, 1975). The duplicate copy of the *gal* structural genes is believed to be fused to a new promoter so that enzyme synthesis is constitutive. The *gal*$^-$ seg-

regants arise by internal recombination and, therefore, their appearance depends upon a functional *rec* system. This proposal offers no explanation for the *recA* independent unstable revertants of IS*2*.

Direct evidence in favor of either hypothesis has not been available. Since the instability in the majority of these reversions is independent of the *recA* function (Morse & Pollock, 1969), it has generally been assumed (for example, see Cohen, 1976; Kolata, 1976) that these revertants arise by inversion of the IS*2*. The orientation of the IS*2* remains unchanged in an unstable constitutive reversion of *gal3 recA*$^-$ studied here, and the instability is interpreted as being due to a high mutation rate generated by errors during the repair of specific lesions formed along IS*2* during transcription. The possibility that this transcription initiates within IS*2* is discussed in the light of experiments involving adenyl cyclase (*cya*) and cyclic AMP receptor protein (*crp*) mutants.

MATERIALS AND METHODS

Bacterial and bacteriophage strains

The genotypes and sources of the bacterial strains used in this study are described in Table 1. All are derivatives of *Escherichia coli* K12. The thermoinducible derivative of bacteriophage λ, λ*c*I857 and the generalized transducing phage P1*vir* were obtained from the collection of Dr. A. Ahmed.

Media

The medium of Davis and Mingioli (as described by Roth, 1970) containing 0.2% glucose, rhamnose, arabinose, maltose, lactose, 1.0% galactose or 2% glycerol was used as the minimal medium. This medium was supplemented with 20 µg/ml of required L-amino acids and 10 µg/ml of thiamine HCl. Streptomycin was added to a concentration of 100 µg/ml after autoclaving when necessary. L broth or L agar supplemented with 50 µg/ml thymine was generally used as the complete medium.

EMB, MacConkey and tetrazolium indicator plates were used extensively throughout this study. The composition and preparation of these plates are described by Miller (1972).

Construction of strains

The basic *gal3* strain used was F^- *thr leu lac gal3* sup^+_{am} *thi* λ^- (Morse, 1972). *gal3*(λ) *strA* was mated with HfrKL16-99 to

TABLE 1 - Bacterial strains

Strain	Genotype	Source/Origin
gal3	F$^-$ *thr leu lac gal3* sup^o_{am} *thi* λ^-	Morse *et al.* (1956)
gal3(λ)	F$^-$ " " " " " " (λcI857)	Lysogenization (AA)**
gal3(λ) *strA*	F$^-$ " " " " " " *strA thi*	Spontaneous *strA* (AA)
Strain 21	F$^-$ *gal3* sup^o_{am} (λcI857) *recA strA thi*	KL16-99 x *gal3*(λ) *strA*
gal^{c}331 recA	F$^-$ *gal^{c}331* sup^o_{am} (λcI857) *recA strA thi*	Spontaneous (AA)
F'108/*gal^{c}331 recA*	F'*recA*$^+$/*gal^{c}331* sup^o_{am} (λcI857) *recA strA thi*	Spot mating (AA)
HMS 83-1	F$^-$ *leu polB100 lacZ thy lys polA1 rha strA*	Campbell *et al.* (1974)
HMS 83-1 Δ*gal*	F$^-$ " " " Δ(*gal-chlD*) *thy lys polA1 rha strA*	Spontaneous deletion
gal^{c}331 polA$^-$ polB$^-$	F$^-$ " " " *gal^{c}331 thy lys polA1 rha strA*	HMS 83-1 Δ*gal* + P1/*gal^{c}331*
F'14/*gal^{c}331 polA$^-$ polB$^-$*	F'*polA*$^+$/*leu polB100 lacZ gal^{c}331 thy lys polA1 rha strA*	Spot mating
F'104/ " " "	F'*polB*$^+$/ " " " " " " " " "	Spot mating
gal^{c}331 polA$^+$ polB$^-$	F$^-$ *leu polB100 lacZ gal^{c}331 thy lys strA*	KL209 x *gal^{c}331 polA$^-$ polB$^-$*
F'104/*gal^{c}331 polA$^+$ polB$^-$*	F'*polB*$^+$/*leu polB100 lacZ gal^{c}331 thy lys strA*	Spot mating
Δ303	F$^-$ *thr leu lac* Δ(*gal-pgl*) sup^+_{am} (λcI857) *thi*	Spontaneous from *gal3*(λ)
EJ331	F$^-$ " " " *gal^{c}331* sup^+_{am} (λcI857) *thi*	Δ303 + P1/*gal^{c}331*

TABLE 1 - continued

Strain	Genotype	Source/Origin
JC5519	F⁻ *thr ara leu proA lacY tsx supE galK his recB21 recC22 strA xyl mtl argE thi* λ⁻	A.J. Clark
EJ331 *thy*⁻	F⁻ *thr leu gal*^{c}*331* sup^{+}_{am} (λ*c*I857) *thyA thi*	Spontaneous from EJ331
gal^{c}*331 recB*⁻ *recC*⁻	F⁻ " " " " " *recB21 recC22 thi*	GJ331 *thy*⁻ + P1/JC5519
N47	HfrH *proB gal relA thi*	A. Ahmed
HfrH *gal*^{c}*331*	HfrH *proB gal*^{c}*331 relA thi*	N47 + P1/*gal*^{c}*331*
N2672	Hfr (O-*thyA-argA-pts*) *lig ts7 thi*	Gottesman *et al.* (1973)
Δ303-2	F⁻ *thr leu lac* Δ(*gal-pgl*) sup^{+}_{am} *thi* λ⁻	Curing Δ303
RL331	F⁻ " " " *gal*^{c}*331* sup^{+}_{am} *thi* λ⁻	Δ303-2 + P1/*gal*^{c}*331* (RL)
RL331 *strA*	F⁻ " " " " " *strA thi* λ⁻	RL331 + P1/AB1157
RL331 *thy*⁻ *strA*	F⁻ " " " " " *thyA strA thi* λ⁻	Spontaneous mutation
RL331 *lig ts7*	F⁻ " " " " " *lig ts7 strA thi* λ⁻	N2672 x RL331 *thy*⁻ *strA*
T94A	F⁺ *bio phe mutT1*	B.J. Bachmann
gal^{c}*331 mutT1*	F⁻ *thr mutT1 lac gal*^{c}*331* sup^{+}_{am} *thi* λ⁻	RL331 + P1/T94A
Stable 3	F⁻ *thr leu lac gal*⁺ sup^{+}_{am} (λ*c*I857) *thi*	Spontaneous from *gal3*(λ) (AA)

TABLE 1 - continued

Strain	Genotype	Source/Origin
EJ200	F⁻ *thr leu lac gal^{c}200 sup$^{+}_{am}$* (λcI857) *thi*	Spontaneous from *gal3*(λ)
Stable 3 λ⁻	F⁻ " " " *gal^{+} sup$^{+}_{am}$ thi* λ⁻	Curing stable 3
EJ200 λ⁻	F⁻ " " " *gal^{c}200 sup$^{+}_{am}$ thi* λ⁻	Curing EJ200
HfrH 81.2	HfrH *gal0^{c}81.2 sup$^{+}_{am}$ thi* λ⁻	G. Buttin
RL81.2	F⁻ *thr leu lac gal0^{c}81.2 sup$^{+}_{am}$ thi* λ⁻	Δ303-2 + P1/HfrH 81.2 (RL)
Stable 3 λ⁻ *strA*	F⁻ " " " *gal^{+} sup$^{+}_{am}$ strA thi* λ⁻	Stable 3 λ⁻ + P1/AB1157
Stable 3 λ⁻ *strA metE*	F⁻ *lac gal^{+} sup$^{+}_{am}$ strA metE thi* λ⁻	KL25 metE x stable 3 λ⁻ *strA*
LS853	F⁻ *trpA his Δ(cya) trpR* λ⁻	B.J. Bachmann
Stable 3 λ⁻ *strA cya*⁻	F⁻ *lac gal^{+} sup$^{+}_{am}$ strA Δ(cya) thi* λ⁻	Stable 3 *metE* + P1/LS853
Stable 3 λ⁻ *strA cya*⁺	F⁻ " " " " *thi* λ⁻	Stable 3 *metE* + P1/LS853
EJ200 λ⁻ *strA*	F⁻ *thr leu lac gal^{c}200 sup$^{+}_{am}$ strA thi* λ⁻	EJ200 λ⁻ + P1/AB1157
EJ200 λ⁻ *strA metE*	F⁻ *lac gal^{c}200 sup$^{+}_{am}$ strA metE thi* λ⁻	KL25 *metE* x EJ200 λ⁻ *strA*
EJ200 λ⁻ *strA cya*⁻	F⁻ " " " " *Δ(cya) thi* λ⁻	EJ200 *metE* + P1/LS853
EJ200 λ⁻ *strA cya*⁺	F⁻ " " " " *thi* λ⁻	EJ200 *metE* + P1/LS853
RL331 *strA*	F⁻ *thr leu lac gal^{c}331 sup$^{+}_{am}$ strA thi* λ⁻	RL331 + P1/AB1157

TABLE 1 - continued

Strain	Genotype	Source/Origin
RL331 *strA metE*	F$^-$ *lac gal^{c}331 sup$^+_{am}$ strA metE thi* λ^-	KL25 *metE* x RL331 *strA*
RL331 *strA cya*$^-$	F$^-$ " " " " Δ(*cya*) *thi* λ^-	RL331 *metE* + P1/LS853
RL331 *strA cya*$^+$	F$^-$ *lac gal^{c}331 sup$^+_{am}$ strA thi* λ^-	RL331 *metE* + P1/LS853
RL81.2 *strA*	F$^-$ *thr leu lac gal0^{c}81.2 sup$^+_{am}$ strA thi* λ^-	RL81.2 + P1/AB1157
RL81.2 *strA metE*	F$^-$ *lac gal0^{c}81.2 sup$^+_{am}$ strA metE thi* λ^-	KL25 *metE* x RL81.2 *strA*
RL81.2 *strA cya*$^-$	F$^-$ " " " " Δ(*cya*) *thi* λ^-	RL81.2 *metE* + P1/LS853
RL81.2 *strA cya*$^+$	F$^-$ " " " " *thi* λ^-	RL81.2 *metE* + P1/LS853
LS854	F$^-$ *trpA his strA* Δ(*crp*) *met trpR* λ^-	B.J. Bachmann
Stable 3 λ^- *strA crp*$^-$	F$^-$ *thr leu lac gal*$^+$ *sup$^+_{am}$ strA* Δ(*crp*) *thi* λ^-	Stable 3 λ^- + P1/LS854
Stable 3 λ^- *strA crp*$^+$	F$^-$ " " " " " " *thi* λ^-	Stable 3 λ^- + P1/LS854
EJ200 λ^- *strA crp*$^-$	F$^-$ " " " *gal^{c}200 sup$^+_{am}$ strA* Δ(*crp*) *thi* λ^-	EJ200 λ^- + P1/LS854
EJ200 λ^- *strA crp*$^+$	F$^-$ " " " " " " *thi* λ^-	EJ200 λ^- + P1/LS854
RL331 *strA crp*$^-$	F$^-$ " " " *gal^{c}331 sup$^+_{am}$ strA* Δ(*crp*) *thi* λ^-	RL331 + P1/LS854
RL331 *strA crp*$^+$	F$^-$ " " " " " " *thi* λ^-	RL331 + P1/LS854
RL81.2 *strA crp*$^-$	F$^-$ " " " *gal0^{c}81.2 sup$^+_{am}$ strA* Δ(*crp*) *thi* λ^-	RL81.2 + P1/LS854

TABLE 1 - continued

Strain	Genotype	Source/Origin
RL81.2 *strA crp*$^+$	F$^-$ *thr leu lac gal0*^{c}*81.2 sup*$^+_{am}$ *strA* Δ(*crp*$^+$) *thi* λ$^-$	RL81.2 + P1/LS854
LS853	F$^-$ *trpA his* Δ(*cya*) *trpR* λ$^-$	B.J. Bachmann
KL25 *metE142*	HfrKL25 *metE142*	A. Ahmed
Stable 3 λ$^-$ *mutT1*	F$^-$ *thr mutT1 lac gal*$^+$ *sup*$^+_{am}$ *thi* λ$^-$	Stable 3 λ$^-$ + P1/T94A
gal3 mutT1	F$^-$ " " " *gal3* " " λ$^-$	*gal3* + P1/T94A
HfrKL16-99	HfrKL16 *recA* λ$^-$	B.J. Bachmann
HfrKL209	HfrJ4 *malB supE* λ$^-$	B.J. Bachmann
46.1	F$^-$ *thr leu lac gal3 sup*$^+_{am}$ (λ*gal*$^+$46.1)(λ*c*I857) *thi*	A. Ahmed
46.1.8A/2	F$^-$ " " " " " (λ*gal3*)(λ*c*I857) *thi*	A. Ahmed
331-TT-I	F$^-$ " " " " " (λ*gal*^{c}*331*)(λ*c*I857) *thi*	λ transduction
Δ303(λ*gal*^{c}*331*)(λ)	F$^-$ " " " Δ(*gal-pgl*) *sup*$^+_{am}$ (λ*gal*^{c}*331*)(λ*c*I857) *thi*	λ transduction
AB1157	F$^-$ *thr ara leu proA lacY tsx supE galK his strA xyl mtl argE thi* λ$^-$	A. Ahmed collection

** The strains marked AA or RL were constructed by A. Ahmed or R. Lee in this laboratory

produce strain 21, a *gal3 recA1*(λ) *strA* derivative. The unstable constitutive reversion *gal^{c}331* was found among *gal$^+$* revertants isolated by plating strain 21 on EMB galactose. The F'108 episome (Low, 1972) was introduced into *gal^{c}331* by spot mating on minimal plates containing 0.4 μg/ml mitomycin C.

A deletion of the *gal* operon of the *polA1 polB100* strain HMS 83-1 (Campbell *et al.*, 1974), was obtained by selecting chlorate resistant mutants (Adhya *et al.*, 1968) and screening for *gal$^-$* mutants on MacConkey galactose. One derivative which simultaneously became *gal$^-$* and chlorate resistant was taken to be the desired deletion strain and designated HMS 83-1 Δ*gal*. *gal^{c}331* was introduced into this background by transducing HMS 83-1 Δ*gal* to *gal$^+$* with P1*vir*. The merodiploids F'*polA$^+$*/*gal^{c}331 polA1 polB100* and F'*polB$^+$*/*gal^{c}331 polA1 polB100* were obtained by transferring the eipsomes F'14 and F'104 (Low, 1972), respectively, into F$^-$*gal^{c}331 polA1 polB100*. F'*polB$^+$*/*gal^{c}331 polA$^+$ polB100* was constructed from a *rha$^+$ polA$^+$ strA* recombinant obtained from the cross HfrKL209 x F$^-$ *gal^{c}331 polA1 polB100*.

EJ331 was obtained by transducing *gal^{c}331* into the *gal-pgl* deletion (Δ303) of *gal3*(λ), with P1*vir*. A thymine-requiring mutant of EJ331 was obtained by the procedure of Miller (1972) modified by the use of 40 μg/ml trimethoprim in minimal plates containing 50 μg/ml thymine. EJ331 *thy$^-$* was transduced to *thy$^+$* with P1*vir* grown on the *recB21 recC22* strain JC5519 to produce *gal^{c}331 recB$^-$ recC$^-$*.

Curing lysogens of λ*c*I857 was routinely done by heat shocking log phase cultures at 42° for seven min, diluting into ice cold broth and chilling on ice for 10 min, growing for 3 generations at 30°, and then plating for temperature resistant colonies at 37°. All manipulations were done in complete medium. Temperature resistant colonies were tested for immunity to λ. Approximately 40% of the survivors of this treatment were sensitive to λ and these were taken as being cured of λ*c*I857. Δ303 was cured in this way to give Δ303-2, a strain allowing transduction of various *gal* markers by P1*vir*, into the genetic background of the original *gal3* strain.

RL331 contains the *gal*^{c}*331* mutation in the *gal3* background. The *strA* allele of AB1157 was introduced by transduction. A thymine requiring mutant was isolated, as described previously, giving RL331 *thy*$^{-}$ *strA*. N2672 *lig ts7* (Gottesman *et al.*, 1973) was mated with RL331 *thy*$^{-}$ *strA* to give *thy*$^{+}$ *strA* recombinants. A recombinant that failed to grow at 41° and which segregated *gal*$^{-}$ colonies at 30° was taken to be *gal*^{c}*331 lig ts7* and designated RL331 *lig ts7*.

RL331, stable 3 λ^{-} and *gal3* were transduced to *leu*$^{+}$ with P1*vir* grown on T94A, a strain carrying Treffer's *mutT1*. Transductants were patched onto the same plates, then onto plates containing streptomycin (100 μg/ml). Derivatives that produced several *strA* mutants per patch were taken to contain the *mutT1* allele.

HfrH *gal*^{c}*331* was constructed by transducing N47 to *gal*$^{+}$

with P1*vir* grown on F'*recA*$^+$/*gal*^{c}*331*.

Stable 3 and EJ200, inducible and stable constitutive revertants, respectively, of *gal3*(λ) have been described earlier (Ahmed & Johansen, 1975). Both of these strains were cured of λ*c*I857 to produce stable 3 λ$^-$ and EJ200 λ$^-$. The *gal0*^{c} mutation 81.2 (Buttin, 1963) from strain HfrH 81.2 was transduced by P1*vir* into Δ303-2 to produce RL81.2.

The *strA* allele of AB1157 was transduced into EJ200 λ$^-$, stable 3 λ$^-$, RL331 and RL81.2 by P1*vir*. Each of these was crossed with KL25 *metE142* and *metE*$^-$ *thr*$^+$ *leu*$^+$ *strA* recombinants were obtained. Only recombinants that were *lac*$^-$ were used for the next step, as they were likely to have retained the *gal* operon of the female strain. EJ200 λ$^-$ *strA metE*, stable 3 λ$^-$ *strA metE*, RL331 *strA metE* and RL81.2 *strA metE* were transduced to *met*$^+$ with P1*vir* grown on the adenyl cyclase (*cya*) mutant LS853 (Brickman *et al.*, 1973). *cya*$^+$ and *cya*$^-$ derivatives of each strain were purified for further study. *cya*$^-$ derivatives were recognized by their inability to utilize rhamnose, arabinose, maltose or glycerol as sole carbon sources.

EJ200 λ$^-$, stable 3 λ$^-$, RL331 and RL81.2 were transduced to streptomycin resistance with P1 grown on the cyclic AMP receptor protein (*crp*) mutant LS854 (Brickman *et al.*, 1973). *crp*$^+$ *strA* and *crp*$^-$ *strA* transductants of each strain were purified for further study. *crp*$^-$ derivatives were recognized by their inability to use rhamnose, arabinose, maltose or glycerol as sole carbon sources.

Sources of DNA for electron microscopy

λgal^{+} and $\lambda gal3$ DNA were obtained from the strains 46.1 and 46.1.8A/2, respectively, described by Ahmed & Scraba (1975). These two λgal genomes are identical except for the presence of IS2 in $\lambda gal3$. $\lambda gal^{c}331$ DNA was obtained from an independent HFT-lysate producing strain (331-TT-1), isolated as a gal^{+} transductant from a λ lysate of $gal^{c}331(\lambda)$. The genetic constitution of this strain is $gal3(\lambda gal^{c}331)(\lambda)$. λgal phages bearing gal^{-} and gal^{wc} segregants of the $gal^{c}331$ reversion were isolated from the strain $\Delta 303(\lambda gal^{c}331)$ (λ). The extended *gal-pgl* deletion on the bacterial chromosome allows rapid identification of colonies of gal^{-} and gal^{wc} segregants arising from $gal^{c}331$ on indicator plates.

Phage preparations and electron microscopy

Lysogens containing the phage needed for heteroduplex analysis were grown to early log phase in L broth supplemented with 50 µg/ml thymine, heat shocked 16 min at 46°, then incubated at 38° with vigorous aeration. Approximately 1 hr after induction, when lysis had begun, $MgSO_4$ was added to a final concentration of 0.01 M. When lysis was complete, chloroform was added to the lysate. After centrifuging in the Sorvall GSA rotor at 13,000 r.p.m. to remove the cell debris, the phage was sedimented in the Spinco #21 rotor at 18,000 r.p.m. for 3¼ hr. The phage pellet was resuspended in 0.01 volume of TMG buffer (0.01 M Tris, 0.01 M $MgSO_4$, and 0.01% gelatin at pH7.4) at 4°. The phage suspension was cleared by low

speed centrifugation in the Sorvall SM24 rotor, then centrifuged to equilibrium in CsCl (Jordan *et al.*, 1968). The upper band, which corresponded to λ*gal* phage in each case, was collected and used for the preparation of heteroduplexes.

DNA heteroduplexes were prepared and mounted for electron microscopy by the formamide technique (Davis *et al.*, 1971). Following uranyl acetate staining, the DNA containing grids were rotary shadowed at 8° with 20 Å of Pt/C. Electron micrographs were taken with a Philips EM300 electron microscope operated at 60 kV. The mean length of λ/λ homoduplexes was 15.39 ± 0.18 μm(=46.5 kb). The lengths of $\lambda gal^{+}46.1$ and λ*gal3* DNA are taken to be 40.1 kb and 41.2 kb, respectively. All length estimates are based on measurements of 8 or more heteroduplexes.

Determination of segregation rates

Overnight cultures of the unstable $gal^{c}331$ reversion typically contain about 15% segregants, interpreted as arising by spontaneous mutations. This unusually large proportion of segregants limits the application of the equation of Luria & Delbruck (1943) for calculating the segregation rates. The following derivation is a modification which can be used for the calculation of high mutation rates (i.e. $>10^{-3}$).

If a unit of time is the average division time divided by ln2, and N_t is the number of bacteria at time t, then

$$dN_t/dt = N_t, \text{ and } N_t = N_o e^{t} \qquad (1)$$

where N_o is the initial number of bacteria in the culture.

Let a be the probability of mutation per bacterium per unit time and ρ_t be the number of mutants at time t. The number of mutants increases because of (i) new mutants arising from mutable cells, and (ii) the multiplication of existing mutants, so

$$d\rho_t/dt = a(N_t - \rho_t) + \rho_t$$

and

$$\rho_t = N_t - (N_o - \rho_o)e^{(1 - a)t} \qquad (2)$$

where ρ_o is the initial number of mutants in the culture. If $F_t = \rho_t/N_t$, the fraction of mutant cells at time t, then by dividing (2) by (1)

$$F_t = 1 - (1 - F_o)e^{-at} \qquad (3)$$

where F_o is the initial fraction of mutant cells. Substituting $t = \ln(N_t\ N_o)$ into (3) and rearranging gives

$$\ln(1 - F_t/1 - F_o) = a\ln(N_o\ N_t) \qquad (4)$$

which can be used to calculate the mutation rate a, if it is high. The mutation rate per bacterium per division is given by $a\ln 2$. It is assumed that the growth rates of the parental strain and all mutants are the same.

F_o, F_t, N_o, and N_t were determined by spreading appropriate dilutions of cultures on indicator plates immediately before, and after, overnight incubation at 30°. For the determination of the effects of 5-bromo-2'-deoxyuridine and UV on segregation frequencies, cultures were treated essentially according to the procedures outlined by Miller (1972). Mitomycin C (0.4 µg/ml) was added to cells

growing in L broth, and removed after 30 min by centrifugation. The cells were resuspended in fresh broth and allowed to grow for several generations in the dark before spreading on indicator plates. The effect of *lig ts7* was determined by growing cells at moderately restrictive temperatures or by heat shocking at non-permissive temperatures followed by growth at the permissive temperature for several generations.

Reversion frequencies were determined by the method of Luria & Delbruck (1943) by plating overnight cultures on selective medium.

Galactokinase assay

Cells for the galactokinase assay were routinely grown at 30° to late log phase in the minimal medium supplemented with 1% casamino acids and 10 µg/ml thiamine HCl and containing 2% glycerol. For the assays involving *cya*$^-$ and *crp*$^-$ strains, glycerol was replaced by 0.4% succinate and 20 µg/ml L-tryptophan was added. The gratuitous inducer D-fucose was added to a concentration of 5×10^{-3} M when required. Cells were harvested by centrifugation, washed with galactokinase extraction buffer (0.01 M potassium phosphate buffer at pH 7.0 containing 1×10^{-3} M β-mercaptoethanol) and stored frozen at -40° as a cell pellet.

For the preparation of cell free extracts, cells were resuspended in galactokinase extraction buffer and disrupted, on ice, by two 30 sec bursts of a Branson S125 sonifier at medium

intensity with a 30 sec cooling period between. The resulting extracts were cleared by centrifugation at 17,000 r.p.m. for 25 min in the Sorvall SM24 rotor. The supernatants were recovered and used directly for the enzyme assays.

Galactokinase activity was assayed by the spectrophotometric method of Heinrich & Howard (1966). The rate of galactose-dependent oxidation of β-NADH was followed by measuring the change in absorbency at 340 nm. Dividing the change in absorbency at 340 nm by 5.65 gives the number of μmoles of galactose phosphorylated. Specific activities are expressed as μmoles of galactose phosphorylated per min per mg of protein. Protein concentrations in the extracts were determined using the Folin-Ciocalteau reagent.

Sucrose gradients for the detection of plasmids

Strains to be tested were grown to early log phase in the minimal medium supplemented with 1% casamino acids, 10 μg/ml thiamine HCl and 0.5 μg/ml thymidine and containing 0.2% glucose and 250 μg/ml 2'-deoxyadenosine. 0.25 mCi of [methyl-^{3}H]thymidine (20 Ci/mmole) was added to each culture and cells were grown to late log phase. Cells were collected by centrifugation and resuspended in 25% sucrose in 0.05 M Tris pH8.0. The cells were lysed by the addition of lysozyme to 1 mg/ml, EDTA to 0.06 M and sodium lauroyl sarcosinate to 1%. NaCl was added to 1 M and the suspensions were stored overnight at 4°. The chromosomal DNA was removed by centrifuging 30 min at 13,700 r.p.m. in the Spinco 50Ti

rotor. The supernatant was collected, mixed with one volume TE buffer (0.01 M Tris, 0.001 M EDTA, pH 8.1) and layered onto a 5 - 20% linear sucrose gradient containing 0.5 M NaCl and 0.01 M potassium phosphate pH 7.0. Centrifugation was done for 60 min at 49,000 r.p.m. in the Spinco SW50L rotor at 15°. Two drop fractions were collected directly into 5 mls Bray's solution and counted in a Beckman LS-230 liquid scintillation counter. This procedure is basically the procedure of Guerry *et al.* (1973).

Genetic techniques

Transductions with the generalized transducing phage P1*vir* were done according to the procedure described by Lennox & Yanofsky (1959). Specialized transductions of the *gal* operon by bacteriophage λ were done by mixing equal volumes of a fresh λ lysate and a fresh overnight culture of the recipient strain in L broth containing 0.01 M Mg^{++}, incubating 20 min at 30° and plating on EMB-galactose plates. *gal*$^{+}$ papillae appear after 2 - 3 days at 30°.

F' plasmid transfers and Hfr matings were performed according to the procedures described by Miller (1972).

RESULTS

Isolation of gal^{c}331

In order to eliminate unstable reversions which might be caused by tandem duplications, *gal*$^{+}$ revertants were selected from a *gal3 recA*$^{-}$ (λ) strain on EMB-galactose. An unstable constitutive reversion, designated *gal*^{c}*331*, was found among 360 revertants tested. This reversion synthesizes galactokinase in a constitutive manner at 36% of the level of a fully-induced wild type. The reversion retains the *recA* mutation, and produces *gal*$^{-}$ segregants at a rate of 8.8×10^{-3} segregants/cell/division. In every respect, *gal*^{c}*331* appears to be a typical representative of the class of unstable reversions of *gal3* described by Morse & Pollock (1969).

Properties of segregants

Previous studies have shown that unstable reversions of *gal3* produce segregants which are identical to the original *gal3* mutation (Morse, 1967; Morse & Pollock, 1969). The same appears to be true for the unstable revertants of *gal308* (Saedler *et al.*, 1974). Segregants of *gal*^{c}*331*, appearing as red colonies on tetrazolium-galactose plates, were tested on EMB-galactose. It was found that these segregants were not uniformly *gal*$^{-}$, but represented a heterogeneous group. About 83% of the segregants were identical to *gal3* in that they were unambiguously *gal*$^{-}$ on EMB-galactose, showed no detectable galactokinase activity, and reverted to produce the three characteristic kinds of revertants at a rate similar

of the original *gal3* strain. The remaining 17% of the segregants appeared to be weak gal^+ on EMB-galactose. Initially, these segregants appear gal^-, but slowly reveal their gal^+ phenotype after 2-3 days incubation at 30°. Under comparable conditions, gal^+ (inducible) and gal^c (constitutive) strains produce a positive response after overnight incubation. These segregants were found to be weak-constitutive (gal^{wc}) and unstable. Their galactokinase activities ranged from 1-20% of the induced wild type level, and their rate of segregation was approximately 8.8×10^{-4} gal^-/cell/division. The production of gal^{wc} segregants is not unique to gal^c331, because subsequent tests showed that R2, an unstable reversion described by Morse (1967), also produces similar segregants. The wide range of enzyme activities exhibited by the gal^{wc} segregants suggests that they might originate by independent mutational events.

The frequencies of appearance of various kinds of revertants from the *gal3* mutation, and their subsequent segregation behavior, are summarized in Figure 1.

Tests for plasmids

The segregation frequency of gal^c331 was not affected by growth in the presence of acridine orange or sodium dodecyl sulfate, treatments which are known to block replication of certain plasmids (Takahashi & Matsubara, 1972). Similarly, centrifugation of [^{3}H]-thymidine-labelled supernatant fluids from gal^c331 cells in neutral sucrose gradients (Guerry *et al.*, 1973) did not reveal the presence

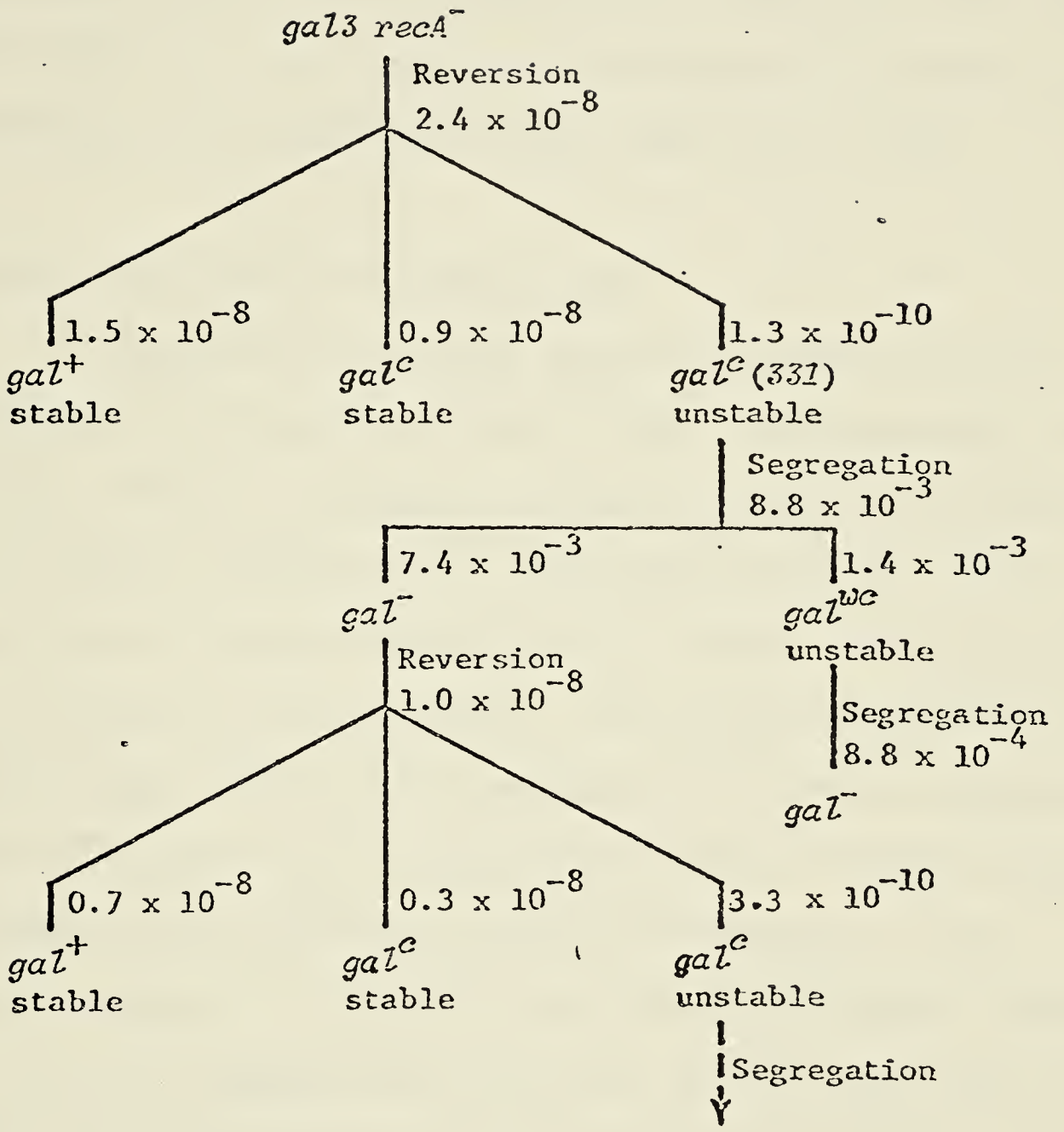


Figure 1. Segregation behavior of *gal^c331*, an unstable constitutive reversion derived from the *gal3* mutation. The various phenotypes are: *gal⁺*, inducible; *gal^c*, constitutive; and *gal^wc*, weak-constitutive. The rates are expressed as revertants (or segregants)/cell/division. In the presence of *recA⁺*, the rate of *gal3* reversion was 1.8×10^{-8}.

of plasmid DNA. Under these conditions, a strain harboring F'103 exhibited a distinct peak of plasmid DNA. These results show that the instability is not caused by the attachment of *gal* genes to an extrachromosomal plasmid-like element (Ames *et al.*, 1963).

Isolation of a gal^{c}331 *transducing phage*

Constitutive reversions of *gal3* are known to block the production of λ*gal* particles (Ahmed, 1977). As a consequence, it has been difficult to construct permanent transducing lines bearing such reversions (Saedler *et al.*, 1974; Ahmed & Johansen, 1975). Heat-induced λ lysates from *gal^{c}331* (λ), however, did transduce a *gal3* recipient at a low frequency. The transductants, which had inherited the original *gal^{c}331* reversion by all criteria, produced normal HFT-lysates containing *gal^{c}331* particles. A lysate from one of these HFT-lines (331-T1-I) was used to transduce a *gal-pgl* deletion (*Δ303-2*) to produce the strain *Δ303*(λ*gal^{c}331*)(λ). Several *gal^{-}* and *gal^{wc}* segregants arising on the λ*gal^{c}331* genome were isolated by plating this heterogenote on tetrazolium-galactose. Only those segregants which were *pgl^{+}* were tested further for *gal^{-}* or *gal^{wc}*, since these had retained the λ*gal^{c}331* genome.

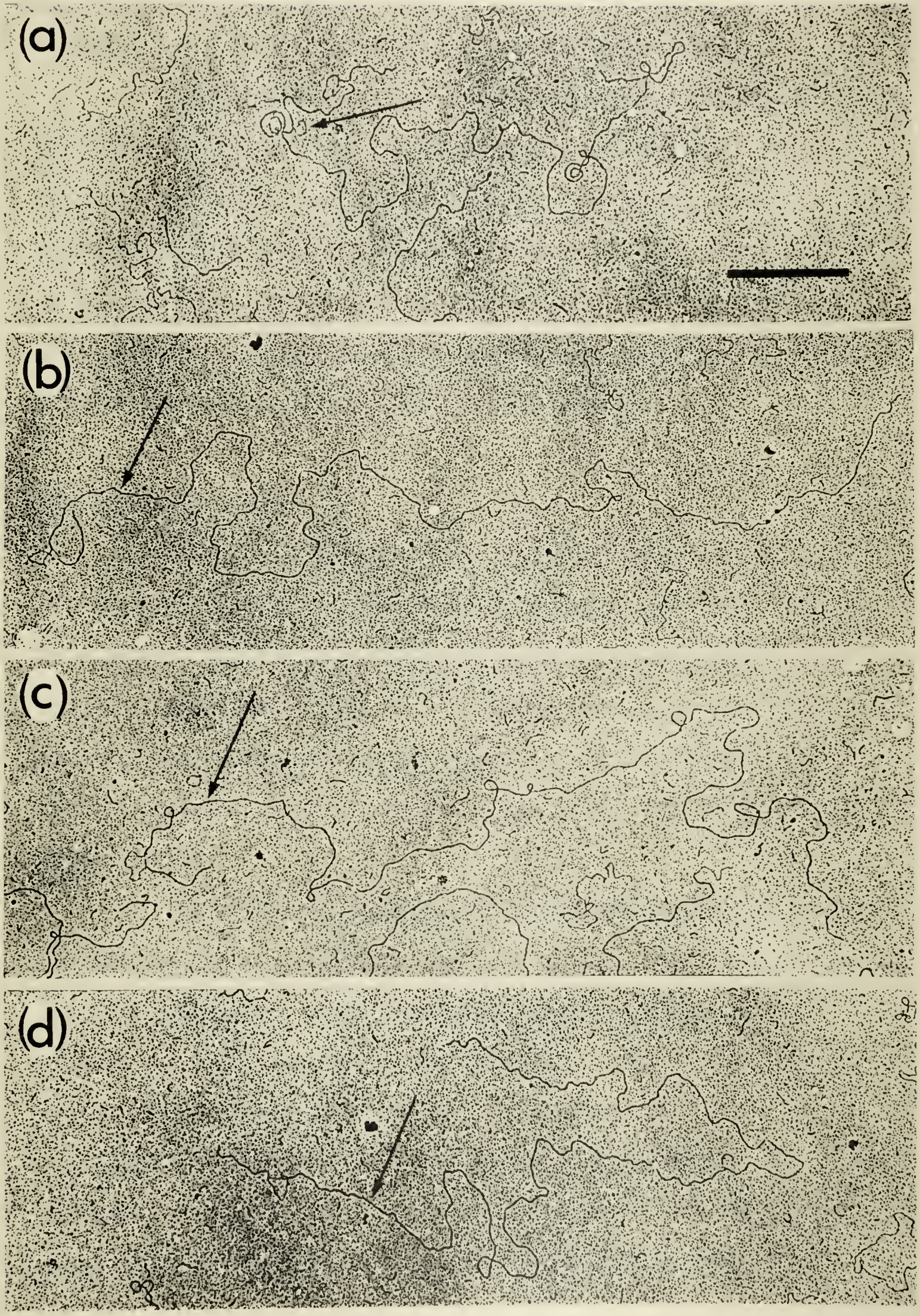
Electron microscopy of the gal^{c}331 *reversion and its segregants*

The presence of IS2 in *gal^{c}331* was verified by electron microscopy of heteroduplexes of λ*gal^{c}331* with reference λ*gal^{+}* DNA, a physical map of which has been published earlier (Ahmed & Scraba,

1975). The overall length of the heteroduplex (Fig. 2a) is 40.1 ± 1.3 kb. It shows extensive sequence homology with only two small loops located near the left end. The left substitution loop is caused by the different points of junction of phage and bacterial DNA in the two λ*gal* genomes. The insertion loop to the right is identified as IS*2*, situated on the λ*gal*^{c}*331* strand, on the basis of its size and location. Starting from the left end, the length measurements on the individual segments are: region of homology at the left, 1.9 ± 0.0 kb; single-stranded arms of the substitution loop, 0.3 ± 0.0 kb and 1.8 ± 0.1 kb; region of homology between the two loops (which includes the *gal* operon), 3.5 ± 0.1 kb; insertion loop (IS*2*), 1.10 ± 0.14 kb; and remaining region of homology up to the right end, 34.4 ± 1.2 kb. The length of the IS*2* is in agreement with our previous estimates on *gal3* and, therefore, the *gal*^{c}*331* reversion retains the entire insertion element.

The orientation of IS*2* in *gal*^{c}*331* was determined from λ*gal*^{c}*331*/ *gal3* heteroduplexes (Fig. 2b). λ*gal3* is identical in sequence to *gal*$^{+}$, except that it harbors an IS*2* element (i.e. the *gal3* mutation) in the polar orientation (I). This heteroduplex resembles the previous example closely, with the exceptions that the overall length is 41.2 ± 0.7 kb and the IS*2* loop is missing. This is because the IS*2* elements present on the two interacting strands are perfectly paired. It can be concluded that, within the limits of resolution of the heteroduplex technique (50 base-pairs), the IS*2* element in *gal*^{c}*331* is identical in size, location, and

Figure 2. Heteroduplexes of $\lambda gal^{c}331$ with (a) λgal^{+} and (b) $\lambda gal3$ reference DNA. Heteroduplexes of gal^{-} and gal^{wc} (weak-constitutive) segregants of $\lambda gal^{c}331$ with $\lambda gal3$ are shown in (c) and (d), respectively. Arrows indicate the location of IS2 expected from the physical map of λgal^{+}. The bar represents 1 μm. From Ahmed & Johansen (1977).


(a)
(b)
(c)
(d)

orientation to that present in the original *gal3* mutation.

DNA heteroduplexes of several gal^- and gal^{wc} segregants of $\lambda gal^c 331$ with $\lambda gal3$ (Fig. 2c and d) were also examined. In each case the configurations and measurements were indistinguishable from the heteroduplex shown in Figure 2b. Therefore, the genetic events on IS*2* which are responsible for the production of segregants are also below the resolution of the heteroduplex technique, and possibly involve alterations of only one or a few base-pairs.

Effect of DNA repair

Since the segregants from $gal^c 331$ do not show any gross alteration by electron microscopy, it is conceivable that they arise by a process similar to mutation during the enzymatic repair of single-stranded gaps along IS*2*. Frequent appearance of nicks and gaps has been postulated as the basis for the formation of IS*2*-specific deletions and inhibition of the production of λgal particles (Ahmed, 1977).

The effect of mutations in various DNA repair functions on the instability of $gal^c 331$ is shown in Table 2. The results show that the segregation rates are significantly increased in the presence of $recA^-$ and $recB^-$ $recC^-$ mutations. The rate is reduced in a strain lacking both the *polA* and the *polB* functions. It is restored by introduction into the strain of a functional $polA^+$ gene, but not by the $polB^+$ gene. This result suggests that DNA polymerase I, coded by *polA*, may play a role in the expression of instability.

TABLE 2 - Effect of DNA repair mutations on the instability of *gal^{c}331*

Strain	Relevant genotype	Segregation rate ($\times 10^3$)
F' recA$^+$/gal^{c}331 recA1	*gal^{c}331*	1.5
F$^-$ gal^{c}331 recA1	*gal^{c}331 recA$^-$*	8.8
F$^-$ gal^{c}331 recB21 recC22	*gal^{c}331 recB$^-$ recC$^-$*	10.2
F$^-$ gal^{c}331 polA1 polB100	*gal^{c}331 polA$^-$ polB$^-$*	3.0
F' polB$^+$/gal^{c}331 polA1 polB100	*gal^{c}331 polA$^-$*	2.5
F' polA$^+$/gal^{c}331 polA1 polB100	*gal^{c}331 polB$^-$*	6.0
F' polB$^+$/gal^{c}331 polA$^+$ polB100	*gal^{c}331*	8.8

Segregation rates are expressed as segregants/cell/division. Due to differences in genetic backgrounds, control rates for the two sets are given separately.

The involvement of DNA repair is most obvious from experiments on the effects of various treatments known to stimulate repair. The segregation rate of a control *HfrH gal^{c}331 recA^{+}* λ^{-} strain was 2.6×10^{-3} segregants/cell/division. Irradiation of this strain with a low dose of UV (120 ergs/mm^{2}) caused a 5-fold increase in the rate of segregation. Addition of mitomycin C (0.4 µg/ml) caused a 2.6-fold increase. Similarly, treatment of this strain with bromodeoxyuridine (600 µg/ml) was found to cause a 2.5-fold increase above the control.

The segregation rate of a *gal^{c}331 lig ts7* double mutant was determined at 30°, 32.5°, 35.5° and 37.5°. As shown in Table 3, the segregation rate at 30° was 1.5×10^{-3} segregants/cell/division. An increase to 1.8×10^{-3} segregants/cell/division was observed at 32.5° but is not considered to be significant. At 35.5°, the segregation rate increases to 3.5×10^{-3} segregants/cell/division, a 2.3-fold increase over the rate at 30°. At 37.5°, viability is much reduced and the segregation rate is increased 3.4-fold to 5.1×10^{-3} segregants/cell/division. The parental *lig^{+}* strain does not show the same increase in segregation rate at high temperature. No change in the rate of mutation to trimethoprim resistance was found in *gal^{c}331 lig ts7* at the various temperatures.

Exponentially growing cultures of *gal^{c}331 lig ts7* containing 6×10^{5} cells/ml were treated at 40° for 120, 180 and 210 min, then transferred to 30° and grown to saturation to allow segregation of *gal^{-}* and *gal^{wc}*. The segregation rate in the control culture

TABLE 3 - Effect of incubation temperature on the segregation rate of *gal*^{c}*331* in the presence of *lig ts7* mutation

Strain	Incubation temperature	Segregation rate (x10^{3})[a]
RL331 *strA lig ts7*	30°	1.5
	32.5°	1.8
	35.5°	3.5
	37.5°	5.1
RL331 *strA lig*$^{+}$	30°	1.3
	37.5°	2.0

[a] Expressed as segregants/cell/division

maintained throughout at 30° was 1.6×10^{-3} segregants/cell/division. Treating an aliquot of this culture for 120 min at 40° caused a slight increase in the segregation rate to 2.1×10^{-3} segregants/cell/division. A 180 min pulse resulted in a 3.1-fold increase to 5.0×10^{-3} segregants/cell/division, while a 210 min pulse increased the segregation rate 5.0 times to 8.1×10^{-3} segregants/cell/division.

Effect of mutT1

The rate of reversion from *gal3* to gal^+, based on 5 independent samples, was 3.98×10^{-8} revertants/cell/division. The reversion rate of *gal3 mutT1*, based on 5 independent samples, was 4.65×10^{-8} revertants/cell/division. Thus, *mutT1* does not enhance the rate of reversion of the *gal3* mutation. The reason for the discrepancy between the reversion rate of *gal3* given here and that in Fig. 1 is probably the small sample size used. The frequency of appearance of unstable revertants of *gal3* was not increased by *mutT1*.

The effect of *mutT1* on $gal^c 331$ instability and on the mutation rate from gal^+ to gal^- in stable 3 λ^- was also determined. The appearance of gal^- mutants from stable 3 λ^- and stable 3 λ^- *mutT1* was followed by plating on minimal plates supplemented with 20 µg/ml L-threonine, 20 µg/ml L-leucine and 10 µg/ml thiamine HCl containing 2% glycerol and 1 µg/ml 2-deoxy-D-galactose as described by Alper & Ames (1975).

No effect was found on the instability of *gal^{c}331*. The rate of mutation from *gal^{+}* to *gal^{-}* increased from 9.35 x 10^{-7} mutants/cell/division in stable 3 λ^{-} to 1.88 x 10^{-4} mutants/cell/division in stable 3 λ^{-} *mutT1*, a 200-fold increase.

Effect of catabolite repression on gal *transcription*

Since transcription of IS*2* seems to be a requirement for instability (see Discussion), it was desirable to learn where this transcription is initiated. The *gal* operon is under catabolite repression *in vivo* but to a much lesser extent than other catabolite repressible operons (Rothman-Denes *et al.*, 1973). Rak (1976) concluded that the transcription of the *gal* operon in constitutive revertants of *gal308* must be initiated at a promoter located within the IS-element. He found that the mRNA is transcribed from IS*2* DNA in the same orientation as the IS*2* in *gal308* as predicted from the present study, and not in the opposite orientation as predicted by Saedler *et al.*, (1974). There is no reason to suspect that the promoter on IS*2* is subject to catabolite repression. Thus, the effect of *cya^{-}* and *crp^{-}* mutations on *gal^{c}331* transcription would indicate whether transcription initiates within IS*2* or at the *gal* operator-promoter. The results of the galactokinase assay of various *cya^{-}* and *crp^{-}* strains are given in Table 4. The enzyme activities of induced *gal^{+}* strains and *galO^{c}* strains decrease on the introduction of *cya^{-}* or *crp^{-}* mutations. The differences in the activities of the uninduced *gal^{+}* strains are not considered to be

TABLE 4 - Galactokinase activities of *cya*⁻ and *crp*⁻ strains

Strain	Relevant Genotype	Galactokinase specific activity[a]
X407	*gal*⁺	0.007
X407 induced[b]	*gal*⁺	0.180
Stable 3 λ⁻ *crp*⁺	*gal*⁺	0.006
Stable 3 λ⁻ *crp*⁻	*gal*⁺ *crp*⁻	0.004
Stable 3 λ⁻ *cya*⁺	*gal*⁺	0.008
Stable 3 λ⁻ *cya*⁻	*gal*⁺ *cya*⁻	0.006
Stable 3 λ⁻ *crp*⁺ induced	*gal*⁺	0.080
Stable 3 λ⁻ *crp*⁻ induced	*gal*⁺ *crp*⁻	0.023
Stable 3 λ⁻ *cya*⁺ induced	*gal*⁺	0.074
Stable 3 λ⁻ *cya*⁻ induced	*gal*⁺ *cya*⁻	0.039
RL81.2 *crp*⁺	*galO*^{c}*81.2*	0.148
RL81.2 *crp*⁻	*galO*^{c}*81.2 crp*⁻	0.075
RL81.2 *cya*⁺	*galO*^{c}*81.2*	0.118
RL81.2 *cya*⁻	*galO*^{c}*81.2 cya*⁻	0.065
EJ200 λ⁻ *crp*⁺	*gal*^{c}*200*	0.116
EJ200 λ⁻ *crp*⁻	*gal*^{c}*200 crp*⁻	0.207
EJ200 λ⁻ *cya*⁺	*gal*^{c}*200*	0.107
EJ200 λ⁻ *cya*⁻	*gal*^{c}*200 cya*⁻	0.225

TABLE 4 - continued

Strain	Relevant Genotype	Galactokinase specific activity[a]
RL331 *crp*$^+$	*gal*^{c}*331*	0.053
RL331 *crp*$^-$	*gal*^{c}*331 crp*$^-$	0.122
RL331 *cya*$^+$	*gal*^{c}*331*	0.051
RL331 *cya*$^-$	*gal*^{c}*331 cya*$^-$	0.085
LS853	*gal*$^+$ *cya*$^-$	0.002
LS853 induced	*gal*$^+$ *cya*$^-$	0.028
LS854	*gal*$^+$ *crp*$^-$	0.008
LS854 induced	*gal*$^+$ *crp*$^-$	0.021

[a] Expressed as μmoles galactose phosphorylated per mg protein per min.

[b] Induced by growth in the presence of 5×10^{-3} M D-fucose.

All strains were grown as described in Materials and Methods in minimal medium supplemented with 1% casamino acids, 20 μg/ml L-tryptophan and 10 μg/ml thiamine HCl and containing 0.4% succinate as the carbon source.

significant. EJ200 λ^- was believed to be constitutive due to disruption of the operator by the sequences remaining after deletion of 3/4 of IS2 (Ahmed & Johansen, 1975). If this were the case, the constitutive expression of *gal*^{c}*200* should be decreased by the introduction of *cya*$^-$ or *crp*$^-$ mutations. As shown in Table 3, the enzyme activity actually increases in the absence of the catabolite repression system. Thus, *gal*^{c}*200* is not constitutive by disruption of the *gal* operator but more likely due to the action of a promoter on the IS2. The enzyme activity of *gal*^{c}*331* also increases on the introduction of adenyl cyclase or cyclic AMP receptor protein mutations. Thus, the constitutive transcription observed in *gal*^{c}*331* is also initiated within IS2.

DISCUSSION

Instability of mutations has been traditionally ascribed to plasmid formation (Ames *et al.*, 1963), tandem duplications (Folk & Berg, 1971), and in one case to the inversion of an insertion element (Saedler *et al.*, 1974). None of these schemes provides an adequate explanation for the instability of the *gal^{c}331* reversion. It was not caused by the attachment of *gal* genes to a plasmid because no plasmid DNA could be detected, and the reversion was transduced by λ. The reversion is not a tandem duplication because the instability is not dependent on the *rec* function, and no loop showing a variable location (Busse & Baldwin, 1972) was detected by electron microscopy. The inversion hypothesis of Saedler *et al.* (1974) is eliminated because the heteroduplexes do not show the characteristic symmetrical loop or bubble expected for an inversion. A small inversion within IS2 (Starlinger & Saedler, 1976), caused by short inverted repeats, also appears unlikely because it would be expected to produce uniform *gal*$^-$ segregants (as would duplications and inversions of IS2). Therefore, it can be concluded that the basis for the instability of *gal^{c}331* is different.

We have shown that the unstable reversions of *gal3* arise at a low ($\simeq 10^{-10}$) frequency, exhibit low levels of constitutive enzyme synthesis relative to stable constitutive reversions, and cause no visible change in the size, location, or orientation of the IS2 in the *galOP* region. A similar observation has been reported for an unstable reversion of *gal308* (Saedler *et al.*, 1974).

Although a small genetic aberration (<50 base-pairs) can not be ruled out, the view that the unstable reversions arise by double mutations on IS2 which cause partial inactivation of the rho-sensitive site is favored. A requirement for double mutations to inactivate a sequence normally used for the termination of transcription is not totally unexpected. The observation that *gal3* can be reverted by nitrosoguanidine, but not by aminopurine, bromouracil, or ICR-191E supports this notion, although these revertants have not yet been fully characterized. Therefore, $gal^{c}331$ is believed to retain the complete, albeit mutationally altered, IS2 element which allows the continuation of transcription at a reduced level. In contrast, the stable constitutive reversions of *gal3* are deletions of the rho site and, therefore, permit high levels of expression.

The most striking feature of $gal^{c}331$ is its instability, i.e. the production of segregants at a high ($\simeq 10^{-2}$) rate. In the past, these segregants were believed to be exclusively gal^{-} (Hill & Echols, 1966; Morse, 1967; Morse & Pollock, 1969). Our results show that these are heterogeneous, and include gal^{-} and a variety of gal^{wc} types. The wide range of kinase activities (1-20% of the wild type) exhibited by the gal^{wc} segregants suggests that they originate from independent genetic events. Since no gross alteration can be seen by electron microscopy, these events are probably mutations occurring at an exceptionally high rate. This conclusion is reinforced by the observation that the gal^{-} segregants spontaneously produce unstable revertants at a relatively high frequency (Fig. 1).

Two essential requirements for this kind of instability are (i) the presence of a specific DNA sequence provided by IS*2*, and (ii) the occurrence of uninterrupted transcription along it. The first requirement is apparent because, out of a large number of *galOP* mutations studied, only two (viz., *gal3* and *308*) have been found to yield unstable revertants. These two are the only *OP* mutations caused by IS*2**. Hence, instability of revertants seems to be an IS*2*-linked phenomenon. Whereas the original insertion mutations are extreme polar and stable, a constitutive reversion (*gal*^{c}*331*) which retains the complete IS*2* element is highly unstable. Therefore, the instability must be introduced by the act of transcription along IS*2*. Constitutive transcription of the *gal* operon alone, as in an O^{c} mutation, is clearly not sufficient. Both conditions must be met in order to generate instability. The specific sequence on IS*2* responsible for the instability must be deleted in the stable constitutive revertants of *gal3*.

There are three possible sites for initiation of *gal* operon transcription in strains containing IS*2* or fragments of IS*2* in the control region of the *gal* operon. According to Hua & Markovitz (1974), the *gal* operon has two operators and two promoters. One operator is the binding site of the *galR* repressor protein. Immediately preceding this operator is a promoter that responds to cyclic AMP and cyclic AMP receptor protein. The other operator and promoter are located such that transcription initiated there must proceed through the *galR* binding site. Presumably, the transcribing

RNA polymerase molecule can displace the *galR* repressor and continue through the structural genes of the *gal* operon. In the absence of cyclic AMP, this would not allow initiation of transcription from the catabolite sensitive promoter. The *capR* (*lon*) gene product is believed to be a repressor molecule that binds to this second operator. There is no indication that transcription from one promoter interferes with transcription from the other. Thus, transcription of the *gal* operon can be initiated from two sites in wild type *E. coli* K12. As discussed earlier, transcription in constitutive revertants of the IS2 mutation *gal308* initiates within the insertion sequence (Rak, 1976). Thus, there are three possibilities for the site of initiation of transcription in gal^c331.

Since the galactokinase activities of gal^c331 and gal^c200 are not decreased by cya^- or crp^- mutations, transcription in these strains must not initiate at the catabolite sensitive promoter. Transcription of gal^c331 could not initiate at the other *gal* operon promoter because four independent *chlD-pgl* deletions, all with one endpoint at the right terminus of IS2 (Ahmed & Scraba, 1977), had maintained normal constitutive expression of the *gal* operon in gal^c331. None of these deletions could have retained the *capR* repressible control signals, yet constitutive transcription of the *gal* operon was still occurring. Thus, transcription of gal^c331 must initiate within the IS2.

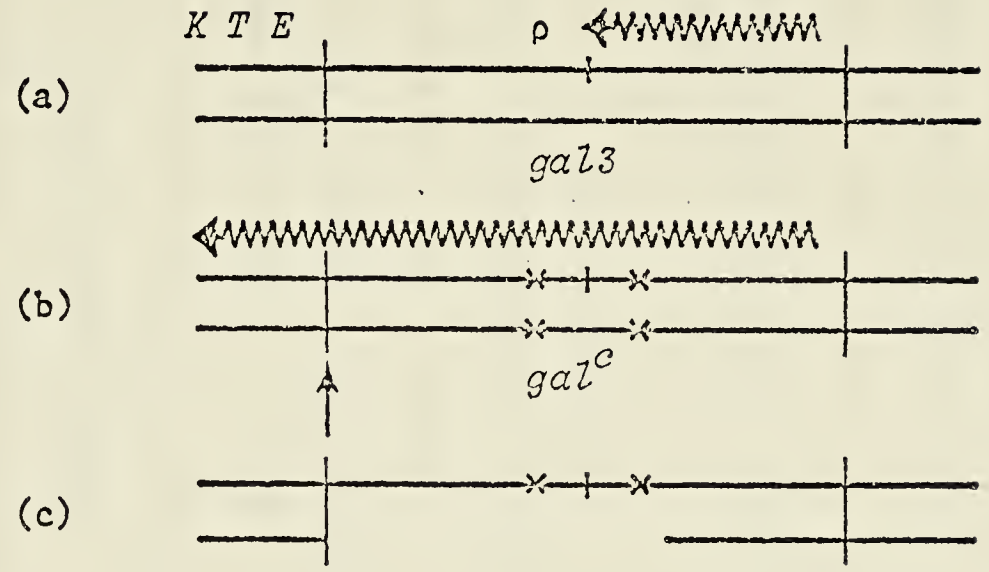
The reason for the increase in galactokinase activities of gal^c revertants of *gal3* in the absence of cyclic AMP or cyclic

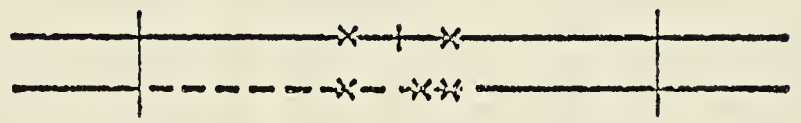
AMP receptor protein is not clear. Possibly the cyclic AMP-receptor protein complex interferes with transcription of the *gal* operon initiated within IS2.

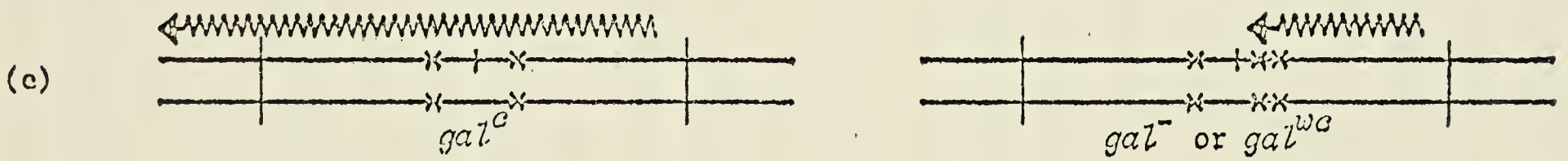
The basis of instability is proposed to be mistakes during repair along IS2, because it appears that these elements can act as hotspots for repair during transcription. As shown in a recent study (Ahmed & Scraba, 1977), these elements act as preferred sites for the formation of extended deletions with one endpoint fixed at IS2. It is suggested that a sequence at the ends of IS2 is nicked by a specific endonuclease, and that these nicks are enlarged exonucleolytically to form the deletions. The frequency of deletions is increased in constitutive revertants, suggesting that the site for nicking is exposed frequently during transcription. The instability of $gal^{c}331$ can be explained by an analogous scheme presented in Figure 3. During transcription, specific nicks appear frequently at the ends of IS2 and are subsequently enlarged into single-stranded gaps. Misincorporation of bases during normal repair of these gaps could cause complete or partial restoration of IS2 polarity. Hence, the reversion would appear to be unstable, and would produce gal^{-} and gal^{wc} segregants at a high frequency.

The above hypothesis, which can generally account for high mutability in localized regions, is supported by preliminary experiments on the effect of DNA repair. UV-irradiation and treatment with mitomycin stimulate repair, and the instability of $gal^{c}331$ is correspondingly increased. The increase observed with bromodeoxy-

Figure 3. A scheme to explain the instability of constitutive reversions of *gal3*. Transcription, initiated at an IS*2* promoter, is terminated at the rho-sensitive site located on IS*2* (a). The unstable reversions (gal^c) arise by double mutations which alter the rho site and allow transcription to proceed through IS*2* into the *gal* genes *E*, *T* and *K* (b). Transcription along IS*2* exposes one end to endonucleolytic attack (↑), and the nick thus created is enlarged into a single-stranded gap (c). Misincorporation of bases during repair (---) leads to the complete or partial restoration of the rho site (d). Following replication and cell division, the gal^c parent produces a variety of gal^- and gal^{wc} segregants at a high rate. Vertical lines denote boundaries of the IS*2* inserted into the *galOP* region.


K T E
ρ
(a)
gal3
(b)
gal^c
(c)


(d)


(e)
gal^c
gal⁻ or gal^wc

uridine is interpreted as being due to the misincorporation of the analog during normal repair of the lesions along IS*2*. The reduction in segregation rate observed with the *polA*$^-$ mutation, and its restoration by the introduction of *F'polA*$^+$, suggests that DNA polymerase I might be involved in the repair process. Since DNA polymerase I has both the exonuclease and polymerase activity postulated to account for instability, this involvement is not surprising. The increased rates observed in *rec*$^-$ mutants seem puzzling at first. Cultures of *rec*$^-$ mutants are known to show low viabilities (18-50%), probably because of their inability to repair the breaks and gaps in the chromosome produced during normal cell growth (Capaldo & Barbour, 1975). The surviving fraction in these cultures must, therefore, consist of cells which have either suffered no damage, or in which the damage has been repaired by an alternative mechanism. Since the *polA*$^-$ *recA*$^-$ and *polA*$^-$ *recB*$^-$ double mutants are known to be completely inviable (Monk & Kinross, 1972), it can be inferred that a high proportion of cells among the survivors in *rec*$^-$ cultures have undergone repair by the alternate pathway. Therefore, it should not be surprising that the instability of *gal*^{c}*331* is increased in *rec*$^-$ mutants.

There are two plausible fates for nicks formed at IS*2* during growth. DNA ligase can immediately reseal the nicks or the nick can be exonucleolytically enlarged to form a gap. This gap would be repaired by polymerase with concomitant errors leading to *gal*$^-$ and *gal*^{wc} segregants, then sealed by ligase. The exonucleolytic degradation and polymerization could be done by one enzyme

leading to nick translation rather than gap formation. The net result is the same. Both mechanisms of dealing with nicks are probably utilized by the cell. Decreasing the amount of functional ligase in the cell by raising the incubation temperature of a temperature sensitive ligase mutant such as *lig ts7* (Gottesman *et al.*, 1973) should favor exonucleolytic degradation over immediate resealing. This is expected to lead to an increase in $gal^{c}331$ instability in a $gal^{c}331$ *lig ts7* double mutant as a higher proportion of the nicks are handled by the segregant producing mechanism. The segregation rate of $gal^{c}331$ *lig ts7* is found to increase as the growth temperature increases.

After two hours at 40°, cell division in *lig ts7* stops (Gottesman *et al.*, 1973), but resumes if the temperature is reduced to 30°. Presumably, the amount of functional ligase is so low after two hours that normal DNA replication and repair cannot occur. Nicks are expected to accumulate during this time. Nick translation, or gap formation and polymerization, would still occur. Thus, a temperature pulse on a $gal^{c}331$ *lig ts7* strain would lead to increased use of the segregant producing mechanism resulting in a higher segregation rate. A temperature pulse of 210 mins results in a 5-fold increase in the segregation rate. Thus, the results obtained with the $gal^{c}331$ *lig ts7* double mutant are in agreement with the hypothesis that nicks occurring at IS*2* are involved in the instability of $gal^{c}331$.

The mechanism of action of *mutT* is not clear. This

mutator gene causes a large increase in the rate of AT → CG transversions (Cox & Yanofsky, 1967). It is not surprising that *mutT1* does not drastically affect the reversion rate of *gal3*, because the vast majority of revertants of *gal3* (>99%) arise by excision of the insertion sequence or deletion of part of the insertion sequence (Ahmed, 1977). Neither of these processes is expected to be enhanced by transversions. Unstable revertants such as gal^c331 are postulated to arise by double mutations within IS2. The *mutT1* allele did not result in an increased frequency of unstable revertants, perhaps because the critical base pairs in the rho-sensitive site(s) on IS2 are GC base pairs (Roberts, 1976), so AT → CG transversions would have little effect. No effect of *mutT1* on gal^c331 was observed, indicating that the mechanism of action of *mutT1* is not involved in gal^c331 instability. The 200-fold increase in mutations from gal^+ to gal^- observed in stable 3 λ^- *mutT1* was not detected in gal^c331 because of the exceptionally high mutation rate in the IS2 region.

In summary, a *recA* independent unstable constitutive revertant of *gal3* is believed to arise by double mutation in the rho-sensitive site on IS2, resulting in a continuation of transcription through the IS-element into the *gal* operon. Transcription of a particular site on IS2 leads to a high rate of endonucleolytic cleavage, possibly at the end of the IS. Errors during the enzymatic repair of the nicks by an exonuclease and a polymerase result in restoration or partial restoration of the rho-sensitive site on

IS2, blocking transcription and resulting in *gal*$^{-}$ or *gal*^{wc} phenotypes. The high frequency of these events is probably due to a high frequency of nicking by the endonuclease during transcription.

FOOTNOTES

† Abbreviations used: kb, kilobase; *gal*^{c} constitutive revertant; *gal*^{wc} weak-constitutive segregant (activity - 20% of a fully-induced wild type).

* The mutation *galE490* was also caused by the insertion of IS*2* within the *OP* region (Reyes *et al.*, 1976; Fiandt *et al.*, 1977). This mutation does not revert.

BIBLIOGRAPHY

Adhya, S. & Shapiro, J. (1969). *Genetics*, 62, 231-247.

Ahmed, A. (1975). *Mol. Gen. Genet.* 136, 243-253.

Ahmed, A. (1977). In *DNA Insertion Elements, Plasmids and Episomes* (Bukhari, A., Shapiro, J. & Adhya, S., eds.), Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Ahmed, A. & Johansen, E. (1975). *Mol. Gen. Genet.* 142, 263-275.

Ahmed, A. & Johansen, E. (1977). *J. Molec. Biol.* Submitted for publication.

Ahmed, A. & Scraba, D. (1975). *Mol. Gen. Genet.* 136, 233-242.

Ahmed, A. & Scraba, D. (1977). *J. Mol. Biol.* Submitted for publication.

Alper, M. & Ames, B. (1975). *J. Bacteriol.* 121, 259-266.

Ames, B., Hartman, P. & Jacob, F. (1963). *J. Mol. Biol.* 7, 23-42.

Brickman, E., Soll, L. & Beckwith, J. (1973). *J. Bacteriol.* 116, 582-587.

Busse, H. & Baldwin, R. (1972). *J. Mol. Biol.* 65, 401-412.

Buttin, G. (1963). *J. Mol. Biol.* 7, 183-205.

Campbell, J., Shizuya, H. & Richardson, C. (1974). *J. Bacteriol.* 119, 494-499.

Capaldo, F. & Barbour, S. (1975). *J. Mol. Biol.* 91, 53-66.

Cohen, S.N. (1976). *Nature* (*London*), 263, 731-738.

Cox, E.C. & Yanofsky, C. (1967). *Proc. Nat. Acad. Sci., U.S.A.* 58, 1895-1901.

Das, A., Court, D. & Adhya, S. (1976). *Proc. Nat. Acad. Sci., U.S.A.* 73, 1959-1963.

Davis, R., Simon, M. & Davidson, N. (1971). In *Methods in Enzymology* (Grossman, L. & Moldave, K., eds.), vol.21, part D, pp. 413-428, Academic Press, New York.

de Crombrugghe, B., Adhya, S., Gottesman, M. & Pastan, I. (1973). *Nature New Biol.* 241, 260-264.

Fiandt, M., Szybalski, W. & Ahmed, A. (1977). *Gene.* In the press.

Folk, W. & Berg, P. (1971). *J. Mol. Biol.* 58, 595-610.

Gottesman, M., Hicks, M. & Gellert, M. (1973). *J. Mol. Biol.* 77, 531-547.

Guerry, P., LeBlanc, D. & Falkow, S. (1973). *J. Bacteriol.* 116, 1064-1066.

Heinrich, M. & Howard, S. (1966). In *Methods in Enzymology.* (Wood, W., ed.), vol.9, pp. 407-412, Academic Press, New York.

Hill, C. & Echols, H. (1966). *J. Mol. Biol.* 19, 38-51.

Hua, S. & Markovitz, A. (1974). *Proc. Nat. Acad. Sci., U.S.A.* 71, 507-511.

Jordan, E., Saedler, H. & Starlinger, P. (1968). *Mol. Gen. Genet.* 102, 353-363.

Kleckner, N. (1977). *Cell,* 11, 11-23.

Kolata, G.B. (1976). *Science,* 193, 392-394.

Lederberg, E. (1960). In *Microbial Genetics* (Hayes, W. & Clowes, R.C., eds.), *X Symp. Soc. Gen. Microbiol.*, pp. 115-131, Cambridge University Press, London.

Lennox, E. & Yanofsky, C. (1959). *Virology*, 8, 425-447.

Low, K. (1972). *Bacteriol. Rev.* 36, 587-607.

Luria, S. & Delbruck, M. (1943). *Genetics*, 28, 491-511.

Miller, J. (1972). *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Monk, M. & Kinross, J. (1972). *J. Bacteriol.* 109, 971-978.

Morse, M. (1967). *Genetics*, 56, 331-340.

Morse, M., Lederberg, E. & Lederberg, J. (1956). *Genetics*, 41, 758-779.

Morse, M. & Pollock, B. (1969). *J. Bacteriol.* 99, 567-569.

Mosharrafa, E., Pilacinski, W., Zissler, J., Fiandt, M. & Szybalski, W. (1976). *Molec. Gen. Genet.* 147, 103-109.

Rak, B. (1976). *Molec. Gen. Genet.* 149, 135-143.

Reyes, O., Gottesman, M. & Adhya, S. (1976). *J. Bacteriol.* 126, 1108-1112.

Roberts, J. (1976). In *RNA Polymerase* (Losick, R. & Chamberlain, M., eds.), pp. 247-272, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Roth, J. (1970). In *Methods in Enzymology*. (Tabor, H. & Tabor, C. eds.), vol.17, part A, pp. 3-35, Academic Press, New York.

Rothman-Denes, L., Hesse, J. & Epstein, W. (1973). *J. Bacteriol.* 114, 1040-1044.

Saedler, H., Reif, H., Hu, S. & Davidson, N. (1974). *Mol. Gen. Genet.* 132, 265-289.

Shapiro, J. & Adhya, S. (1969). *Genetics,* 62, 249-264.

Starlinger, P. & Saedler, H. (1976). In *Current Topics in Microbiology and Immunology,* 75, 111-154.

Takahashi, S. & Matsubara, K. (1972). *Mol. Gen. Genet.* 114, 281-289.

Willetts, N. & Clark, A. (1969). *J. Bacteriol.* 100, 231-239.

B30184